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(54) TUMOR NECROSIS FACTOR ANTAGONISTS AND THEIR USE IN ENDOMETRIOSIS TREATMENT  
TUMOR NEKROSE FAKTOR ANTAGONISTEN UND IHRE VERWENDUNG GEGEN ENDOMETRIOSE  
ANTAGONISTES DE FACTEURS DE NÉCROSIS TUMORALES ET LEUR UTILISATION CONTRE L'ENDOMÉTRIOSE

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(73) Proprietor: Applied Research Systems ARS Holding N.V. Curacao AN	• MORI H.: "Danazol suppresses the production of IL-1 beta and TNF by human monocytes." AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, vol. 24, no. 2, October 1990 (1990-10), pages 45-50, XP022100817
(72) Inventors: • BORRELLI, Francesca I-00179 Roma (IT) • D'ANTONIO, Mauro I-00100 Caserta (IT) • MARTELLI, Fabrizio I-00162 Roma (IT)	• Backett et al.: "Matrix metalloproteinase inhibitors 1998", Exp. Opin. Ther. Patents (1998) 8(3):255-282
(74) Representative: Molnàs, Michel Molnàs & Savoie S.A., 42 rue Plantamour 1201 Genève (CH)	

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Description

Field of The Invention

[0001] Endometriosis factor antagonists are to be administered in therapeutically effective doses to treat and/or prevent endometriosis. The antagonists of this invention typically are selected among various classes of molecules but preferably are soluble TNF receptors. The antagonists are useful for the regression of endometriotic lesions and, for improving the implantation and fertility rate by reducing endometriotic lesions.

Background of The Invention

[0002] Endometriosis is a female genital disease characterized by the presence of endometrial glands and stroma outside of the endometrial cavity and uterine musculature. The anatomical sites most often affected are the ovaries, uterosacral ligaments, pelvic peritoneum, rectovaginal septum, cervix, vagina, the fallopian tubes and vulva. Generally endometriosis is likely to infiltrate deeply from the rectovaginal septum in the underlying tissues and not be visible superficially. Occasionally, foci of endometriosis can be encountered in extratubal sites, i.e. lungs, bladder, skin, pleura and lymph nodes. Endometriotic lesions are progressive, they are first seen as clear vacuoles, which then become red and progress to black, fibrotic lesions over a period of few years (MacSwiney, 1993).

[0003] Endometriosis is considered as a benign disease, but endometriotic lesions become occasionally malignant. As in other kind of malignancies, the development of endometrioma-derived neoplasms is due to concurrent events, involving alterations in growth factors and/or oncogenes regulation (Gronlund, 1996).

[0004] Endometriosis is among the most common gynaecological diseases, with prevalence among reproductive age women this disease is found in about 5-10% of women in reproductive age (Barbieri, 1988). Endometriotic tissue is completely dependent on estrogen for continued growth, also in ectopic locations. Consequently, endometriosis is very sensitive to oestrogen and after oophorectomy, where women are deficient in estrogen, endometriosis hormonal sensitivity is underlying some of the more common symptoms, which are pelvic pain and dysmenorrhea.

[0005] Endometriosis is originated from endometrial cells disseminated from uterus to other locations, where viable cells can implant and grow. Two possible mechanisms have been proposed to explain the initial cell spreading. Retrograde menstruation, a common phenomenon among cycling women, makes possible to endometrium-detached fragments to reach, through menstrual reflux liquid, nearby structures in the genital apparatus. Alternatively, to explain the occurrence of endometriosis in sites other than genital structures, endometrial cells may be spread through uterine veins and extension through the lymphatic system (haemogenous or lymphatic dissemination). Also gynaecological surgery can contribute to this spreading (MacSwiney, 1993).

[0006] Apart from endometrial cell dissemination, other factors, such as genetic predisposition (Malinak et al., 1980), as well as immunological alterations (Ho et al., 1987) may determine women's susceptibility to endometriosis. Since endometrial cells are frequently seen in peritoneal fluid in all women at the time of menses, mammals should have mechanisms, most probably related to immune system, to avoid the onset of endometriosis in general, endometrial cells that escape the host's immune response and have adequate estrogenic stimulation can proliferate to form large, macroscopically visible lesions. Endometriosis is therefore considered as a dynamic process where new lesions are continuously being formed while existing lesions may grow or be destroyed by the host's immune response.

[0007] The inflammatory reaction, normally associated to endometriosis, changes the peritoneal environment, since there is an increased volume of peritoneal fluid and peritoneal macrophages are increased in both number and activity. Therefore, monocyte/macrophage system has been proposed as having a role in the development of endometriosis. Secretory products of macrophages, including cytokines (Shabot et al., 1997), Interleukin-6 (Harada et al., 1997), Interleukin-8 (Shabot et al., 1998), Tumor necrosis Factor- $\alpha$  (Overman et al., 1995), Monocyte Chemoattractant Protein-1 (Avici et al., 1997), were found at higher concentration in the peritoneal fluid of women affected by this disease. Immunological changes have been demonstrated in women with endometriosis but it has not been demonstrated whether those events are responsible for the endometriosis or are a result of the inflammation caused by endometriosis (Rane et al., 1995).

[0008] The knowledge about endometriosis, and its relevance for other disorders, is still now limited, even at diagnostic level. Although endometriosis is considered as a major cause of infertility, studies on the pathophysiology of the disease are contradictory and not definitive. There is a poor correlation between the degree of pain or infertility and the severity of disease, since the early lesions are more metabolically active. The infertility rate is higher than the normal population and studies in rabbits have shown that surgical induction of endometriosis leads to a decrease in fertility from 75% to 25% (Hahn et al., 1988). Patients with pelvic pain were found to have endometriosis 71% of the time, while 84% of patients with pelvic pain and infertility had endometriosis diagnosed (Koninkx et al., 1991). In general, infertility can be found when endometriosis is so extended to disrupt normal vaginal structure, meanwhile pregnancy rates are normal when endometriosis is minimal.

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[0009] Endometriosis can affect fertility also in a different way. White blood cell messengers, like Interleukin-6, Interferon and Tumor Necrosis Factor, are all increased, adversely affecting oocyte-sperm interaction. Serum samples obtained from women with endometriosis were found to be embryotoxic in mouse embryo models and to inhibit sperm mobility in vitro (Haine, 1991), an effect enhanced when recombinant Tumor Necrosis Factor- $\alpha$  is added (Eisermann et al., 1989). Those studies, however, did not address the problem on how cytokines affect the progression of endometriosis but only showed the effects of such molecules on the viability of germ and embryonic cells.

[0010] Hormonal therapy and surgery are the two therapeutic modalities currently used to treat endometriosis. Current pharmacological therapy for endometriosis requires hormonal suppression of the production of estrogen, so that the poor hormone environment blocks the growth of ectopic tissue. Regarding the treatment of endometriosis-related infertility, hormone therapy in patients with minimal disease is of no proven benefit, meanwhile other studies showed an increase in pregnancy rates (Ardi et al., 1996).

[0011] Hormonal therapies have included high dose of progestogens, combinations of estrogen and progestrone (using high dose oral contraceptive pills, or OCPS, in a "pseudopregnancy" regimen), Danazol (an androgenic derivative of ethisterone) and more recently GnRH agonists. These hormonal therapies are effective on pelvic pain and induce an objective regression of lesions, but do not cure the disease. Estrogenic side effects and causing preference of endometriosis to a tissue that is unable to respond to progestogens, even at high doses so that OCPS may offer partial relief to a limited number of patients (Dowdell, 1993). Progestational agents can provoke irregular bleeding (50%) along with depression, weight gain, and fluid retention. Danazol suppresses endometriosis evoking various responses, including the reduction of soluble Tumor Necrosis Factor alpha, Interleukin-1 beta and C9B levels in serum (Matlakidou et al., 1997; Mod et al., 1990), the inhibition of de novo steroidogenesis and the displacement of estradiol from its receptor. Danazol can improve symptoms in approximately 65-100% of the patients suffering from pain, but crude recurrence rates after up to 4 years are approximately 40%-50%. Other drawbacks of Danazol therapy are weight gain and androgenic side effects, which can cause up to 80% of patients to abandon this therapy (Barbieri, 1988). GnRH analogs are more potent and long acting than native GnRH, which work by removing the estrogenic stimulus for the growth of all estrogen sensitive tissues. Side effects of GnRH analogs are mainly secondary to the profound hypoestrogenemia, like decreased bone density, and recurrence rate is up to 50% after 5 years (Walter and Shaw, 1993).

[0012] Depending on the degree of disease, surgical intervention can be conservative, if fertility is desired, or can lead to the removal of the uterus, tubes and ovaries in case of severe disease. In any case, even limited surgical treatment leads to a significant decrease in fertility. Pregnancy rates following surgery generally range between 35% and 65%, so patients need ovulation induction and intrauterine insemination to achieve normal fecundity (Kornicki and Martin, 1994). Clinical reports show that after laparotomy and resection of endometriosis up to 40% of patients required re-operation within 5 years. Even after an aggressive surgical intervention, remnants of pain from endometriosis continue to be a significant problem. Some of the reasons for the failure of surgical therapy may include incomplete resection with lesions either not visible or obscured by the dissection performed. Many lesions are microscopic and would not be able to be detected despite the magnification afforded by the laparoscope. Therefore, surgery alone cannot be considered curative (Devall et al., 1995).

[0013] Many patients with endometriosis suffer from the drawbacks of traditional therapies (including hormonal equilibrium consequences, high recurrence rates and infertility). It is therefore of interest to provide alternative treatments for endometriosis. A possible therapeutic approach may be represented by the use of immunomodulatory molecules which could be able to ameliorate both endometriotic lesions and immunological situation. Such an approach has been considered feasible for the treatment of general symptoms (Rane et al., 1995) but there are no experimental evidences pointing out which cytokine, amongst the ones with altered levels of expression following endometriosis, could be a preferred target for the therapeutic intervention.

[0014] As previously said, one of the several macrophage secretory products involved in endometriotic inflammatory reaction is Tumor Necrosis Factor (abbreviated, from now on, as TNF). TNF, also defined as Cachectin, is a pleiotropic cytokine released by activated T cells and macrophages. TNF is a member of the Interferon, Interleukin and Colony Stimulating Factor cytokine network, which has a key role in signaling system with regard to the pathogenesis of many infectious and inflammatory diseases by inducing a number of proinflammatory changes, including production of other cytokine and adhesion molecule (Fiers, 1991).

[0015] For convenience, the term TNF collectively shall mean, in the entire text of the present application, both Tumor Necrosis Factor- $\alpha$  or - $\beta$  or from animals or humans, together with naturally occurring alleles thereof TNF- $\alpha$  (Pennica et al., 1984), TNF- $\beta$ , also named as lymphotactin, has a similar activity but a broader specificity (Goto et al., 1984).

[0016] TNF is expressed as a mature 17 kDa protein that is active as a trimer. This complex exerts its biological activity by aggregating their surface receptors, which mediate specific effects in different organs and tissues. In endometrium, TNF expression is site- and menstrual cycle-dependent (Hunt et al., 1992), and induces apoptosis in endometrium of experimental animals (Shabot et al., 1999). The adherence of endometrial stromal cells to mesothelial

cells was significantly increased by pre-treatment of mesothelial cells with TNF (Zhang et al., 1993), thus supporting the idea that TNF might contribute to the initiation and/or development of endometriosis.

[0017] TNF exerts its activity, which is required for the normal development and function of immune system, by binding a family of membrane bound receptor molecules including p55 TNF receptor I, defined in the literature also as TNF-R1 and p75 TNF receptor, defined in the literature also as TNF-R2 (Bazan and Beutler, 1998). The dominance of TNF-R1 in transducing TNF signal is suggested by the ability of agonistic antibodies specific for this receptor to mimic the majority of TNF induced responses (Shabot et al., 1998). By using a truncated version of TNF, which does not trigger the signaling pathway through cytosolic activation of TRADD and TRAP-1 (i.e. TNF-R1 or TRAP-1 and TRAF-2) but rather activates the extracellular portion of the receptor, i.e. the T cell proliferation, tumor cell lysis in vitro, dermal neocysts, granuloma formation, apoptosis. The extracellular portion of both TNF receptors can be shed and these soluble receptors retain the ability to bind TNF, neutralizing TNF activity by formation of high affinity complexes, thereby reducing the binding of TNF to target cell membrane receptors (Nophar et al., 1990).

[0018] In endometrium, membrane-bound TNF receptors levels are affected by the administration of oestradiol and/or progestrone, resulting in a temporal and cell type-specific expression of TNF-R1 in mouse (Roby et al., 1996).

[0019] However, this study, like many other studies conducted in various models relates to endometriosis did not give any hints on the real in vivo effect of TNF and TNF-R1, either membrane-bound or soluble, in the development of endometriotic fluid but only a description of the endometriosis-associated immunological abnormalities.

Summary of The Invention

[0020] [0019] Present Patent Application is based on the assumption that e TNF antagonist is able, by sequestering circulating TNF, to block the progression of endometriotic lesions. This assumption is confirmed by the finding reported in the example, showing that a TNF antagonist significantly reduces the size of the endometriotic-like foci in a rat experimental model.

[0021] As a result of applicant's finding, the use of a therapeutically effective amount of TNF antagonist wherein said TNF antagonist is a sequestering or a signaling antagonist is provided herein for the manufacture of a medicament to treat and/or prevent endometriosis in an individual.

[0022] A still further object of the present invention is the use of a TNF antagonist wherein said TNF antagonist is a sequestering or a signaling antagonist together with a pharmaceutically acceptable carrier in the preparation of pharmaceutical compositions for treatment of endometriosis.

[0023] In the present invention, administration of said TNF antagonist can be parenteral or other effective formulations. Any mode of parenteral administration may be suitable including, Intravenous, intramuscular and subcutaneous. Besides the pharmaceutically acceptable carrier, the composition of the invention can also comprise minor amounts of additives, such as stabilizers, excipients, buffers and preservatives.

[0024] TNF antagonists useful in the use of the present invention include soluble TNF receptor molecules, anti-TNF antibodies and compounds which prevent and/or inhibit TNF receptor signaling. It is possible to use the TNF antagonist alone or in combination with other TNF antagonists. The combination with one or more pharmaceutically active products is also possible, in particular to ameliorate conditions of patients suffering from endometriosis-related infertility.

Descriptions of The Invention

[0025] [0023] The invention described herein clearly shows the unexpected result that sequestering TNF (which is only one of the several cytokines whose level of expression is increased in peritoneal fluid following endometriosis), by means of a TNF antagonist, reduces endometriotic-like foci in a rat experimental model. This model demonstrates also that such effect is obtained without affecting significantly the hormonal equilibrium and Natural Killer cells activity. The reduction of endometriotic lesions using TNF antagonists can also improve fertility rates, since the normalization of genital structure has a positive effect on the implantation rate.

[0026] [0024] Therefore, the main object of the present invention is to provide the use of a therapeutically effective amount of TNF antagonist wherein said TNF antagonist is a sequestering or signaling antagonist for the manufacture of medicament for treating endometriosis-related conditions of infertility in an individual.

[0027] In a second embodiment, the invention relates to wherein said TNF antagonist is a sequestering or signaling antagonist for the manufacture of a medicament for treating endometriosis-related conditions of infertility in an individual in combination with other drugs.

[0028] A still further object of the present invention is the use of said TNF antagonist together with a pharmaceutically acceptable carrier in the preparation of pharmaceutical compositions for treatment and/or prevention of endometriosis.

[0029] The active ingredients of the claimed compositions herein are TNF antagonists. Claimed TNF antagonists exert their activity in one of two ways. First, antagonists can bind to or sequester the TNF molecule itself with sufficient affinity and specificity to substantially neutralize the TNF epitope responsible for TNF receptor binding (hereinafter termed "sequestering antagonists"). Alternatively, TNF antagonists can inhibit TNF signaling pathway activated by

cell surface receptor after TNF binding (hereinafter termed "signaling antagonists"). Both groups of antagonists are useful, either alone or together, in the therapy of endometriosis, according to the present invention.

[0022] TNF antagonists are easily identified and tested by routine screening of their effect on the activity of native TNF on susceptible cell lines *in vitro*, for example human cells, in which TNF causes proliferation and Ig secretion. The assay contains TNF formulation at varying dilutions of candidate antagonist, e.g., 0.1 to 100 times the molar amount of TNF used in the assay, and contains no TNF as the only antagonist (Tortorella et al., 1992).

[0023] Sequestering antagonists are the proteins or TNF antagonist according to the present invention. Amongst sequestering antagonists, soluble TNF with high affinity and possess low immunogenicity are preferred. Soluble TNF receptor molecules and neutralizing antibodies to TNF are particularly preferred. For example, TNF-R1 and TNF-R2 are useful in the present invention. Truncated forms of these receptors, comprising the extracellular domains of the receptors or functional portions thereof, are more particularly preferred antagonists according to the present invention. Truncated forms of the TNF receptors are soluble and have been detected in urine and serum as 50 kDa and 40 kDa TNF inhibitory binding proteins, which were originally called respectively TBPI and TBPII (Engelmann et al., 1990). Derivatives, fragments, regions and biologically active portions of the receptor molecules functionally resemble the receptor molecules that can be used in the present invention. Such biologically active equivalent or derivative of the receptor molecule refers to the portion of the said polypeptide, or of the sequence encoding the receptor molecule, that is sufficient size and able to bind TNF with such an affinity that the interaction with the membrane-bound TNF receptor is inhibited or blocked. In a preferred embodiment, human soluble TNF-R1 is the TNF antagonist to be administered to patients. The natural and recombinant soluble TNF receptor molecules and methods of their production have been described in the European Patent Applications EP 308,378, EP 399,327 and EP 433,900 [0024] TNF receptor multimeric molecules and TNF immunoreceptor fusion molecules, and derivatives or portions thereof, are additional examples of receptor molecules useful in the methods of the present invention. TNF receptor multimeric molecules useful in the present invention comprise all or a functional portion of the extracellular domain of two or more TNF receptors linked via one or more polypeptide linkers. The multimeric molecules can further comprise a signal peptide of a secreted protein to direct expression of the multimeric molecule. These multimeric molecules and methods of their production have been described in the European Patent Application EP 526,905.

[0025] TNF immunoreceptor fusion molecules useful in the methods of the present invention comprises at least one portion of one or more immunoglobulin molecules and all or a functional portion of one or more TNF receptors. These immunoreceptor fusion molecules can be assembled as monomers, or trimers or homo-multimers. The immunoreceptor fusion molecules can also be monovalent or multivalent. TNF immunoreceptor fusion molecules and methods for their production have been described in the European Patent Application EP 620,738, corresponding to PCT Patent Application WO 94/06478.

[0026] Another class of sequestering antagonists useful in the method of the present invention is represented by the anti-TNF antibodies, including monoclonal, chimeric, humanized, and recombinant antibodies and fragment thereof which are characterized by high affinity binding to TNF in *vitro* and low toxicity. The antibodies which can be used in the invention are characterized by their ability to treat patients for a period sufficient to have good to excellent regression of endometriotic lesions, alleviation of symptoms and low toxicity. Neutralizing antibodies are readily raised in animals such as rabbits or mice by immunization with TNF. Immunized mice are particularly useful for providing sources of B cells for the manufacture of hybridomas, which in turn are cultured to produce large quantities of anti-TNF monoclonal antibodies. Chimeric antibodies are immunoglobulin molecules characterized by two or more segments or portions derived from different animal species. Generally, the variable region of the chimeric antibody is derived from a non-human mammalian antibody, such as murine monoclonal antibody, and the immunoglobulin constant region is derived from a human immunoglobulin molecule. Preferably, both regions and the combination have low immunogenicity as routinely determined (Elliott et al., 1994). Humanized antibodies are immunoglobulin molecules created by genetic engineering techniques in which the murine constant regions are replaced with human counterparts while retaining the murine antigen binding regions. The resulting mouse-human chimeric antibody should have reduced immunogenicity and improved pharmacokinetics in humans (Knight et al., 1993). Preferred examples of high affinity monoclonal antibodies and chimeric derivatives thereof, useful in the methods of the present invention, are described in the European Patent Application EP 186,833 and PCT Patent Application WO 92/16553.

[0027] TNF antagonist can be administered orally and parenterally in various ways. The routes of administration include intradermal, topical, oral, rectal, parenteral, i.v., parenteral, i.m., parenteral, i.m./parenteral, i.v., parenteral, i.v./parenteral, i.v./topical, and rectal routes. Any other therapeutically efficacious route of administration can be used, for example absorption through epithelial or endothelial tissues or by gene therapy wherein a DNA molecule encoding the TNF antagonist is administered to the patient (e.g. via a vector) which causes the TNF antagonist to be expressed and secreted *in vivo*. In addition, the TNF antagonist can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, excipients, diluents or any other carrier.

[0028] The definition of "pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered.

listed. For example, for parenteral administration, TNF antagonist may be formulated in a unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

[0029] For parenteral (e.g. intravenous, subcutaneous, intramuscular) administration, TNF antagonist can be formulated as a soluble suspension, emulsion or lyophilized form in association with a pharmaceutically acceptable carrier (e.g. polyethylene glycols, salts, preservatives and buffers). The formulation is sterilized by common used techniques.

[0030] The bioavailability of the TNF antagonist can be also enhanced by using conjugation procedures which increase the half-life of the molecule in human body, for example linking the molecule to Polyethylene-glycol, as described in the PCT Patent Application WO 92/13056.

[0031] The therapeutically effective amounts of TNF antagonist will be a function of many variables, including the type of antagonist, the affinity of the antagonist for TNF, any residual cytotoxic activity exhibited by the antagonist, the route of administration, the clinical condition of the patient (including the desirability of maintaining a non-toxic level of endogenous TNF activity), the presence of multiple TNF combining sites in sequestering agents, e.g. antibodies.

[0032] A "therapeutically effective amount" is such that when administered, the TNF antagonist results in inhibition of the biological activity of TNF. The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factor, including TNF antagonist pharmacokinetic properties, the route of administration, patient conditions and characteristics (e.g., age, body weight, health, etc.), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled, as well as *in vitro* and *in vivo* methods of determining the inhibition of TNF in an individual.

[0033] Since the maximum tolerated dose of TNF in human clinical trials has ranged up to about 25 micrograms/m<sup>2</sup> body surface/24 hrs, the amount of antagonist administered generally needs not exceed a dose which is calculated to neutralize this amount of TNF. Accordingly, the molar dose of TNF antagonist will vary about from 0.001 to 10 times the maximum tolerated molar dose of TNF; although not above this will be subject to a great deal of therapeutic discretion.

[0034] Moreover, the data obtained in clinical studies, wherein the increase of the concentration of TNF in peritoneal fluid in women with endometriosis was monitored using various methods (Eulermann et al., 1991; Hahn, 1991; O'Farrell et al., 1994), can be useful in determining the TNF antagonist effective dose to be administered.

[0035] Usually a single dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight, ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage which is the same, less than or greater than the initial or previous dose administered to the individual. A second or subsequent administration can be administered during or prior to relapse of the endometriosis or the related symptom. The terms "relapse" or "recurrence" are defined to encompass the appearance of one or more of symptoms of endometriosis.

[0036] The TNF antagonist can be administered prophylactically or therapeutically to an individual prior to, simultaneously or sequentially with other therapeutic regimen or agents (e.g. multiple drug regimen), in a therapeutically effective amount, in particular for the treatment of infertility. TNF antagonists that are administered simultaneously with other therapeutic agents can be administered in the same or different compositions. In particular, when infertility is the endometriosis associated disorder intended to be cured, biologically active human chorionic gonadotropin (hCG), luteinizing hormone (LH) or follicle stimulating hormone (FSH), either in a natural highly purified or in a recombinant form, can be administered. Such molecules and methods of their production have been described in the European Patent Applications EP 186,639, EP 211,894 and EP 322,438.

[0037] The present invention will now be illustrated by the example, which is not intended to be limiting in anyway, and makes reference to the following figures.

#### 4 Description of The Figures

[0044]

Figure 1 shows the effects of Anti<sub>2</sub> (2 mg/kg, subcutaneous given every 3 days), recombinant soluble TNF-R1 (10 mg/kg s.c. given in two daily doses over a 1-week period) on the size of engraftments in rats with experimental endometriosis 2 days and 6 days after the last treatment. These data, which were obtained using 6 animals/group for the first sacrifice time-point and 5 animals/group for the second sacrifice time-point, represent the mean percentage of inhibition ± SEM (standard Error of measurement).

Figure 2 shows the effects of recombinant soluble TNF-R1 (10 mg/kg s.c. given in two daily doses over a 1-week period) and Anti<sub>2</sub> (2 mg/kg s.c. given every 3 days) on the rat NK activity of rat spleen cells against YAC cells (<sup>51</sup>Cr release) 2 days (panel A) and 9 days after the last treatment (panel B). Data represent mean percentage of lysis ± SEM.

Figure 3 shows the effects of recombinant soluble TNF-R1 (10 mg/kg s.c. given in two daily doses over a 1-week

period) as compared to control and Anti<sub>2</sub> (2 mg/kg s.c. given every 3 days) on estradiol-17 $\beta$  serum levels on experimental endometriosis in rats. Data represent mean concentration of estradiol-17 $\beta$ ±SEM.

#### Example

##### Materials and Methods

[0045] Animals. Female Sprague-Dawley rats (250-275 g) were purchased from Charles River Italia (Calco, Lecce, Italy). The animals were housed under the following environmental conditions: temperature 22±2 °C, relative humidity 55±10%, ventilation 15±3 air changes per hour (filtered on HEPA 99.997% filters and artificial lighting with a circadian cycle of 12 hours of light (7:00-19:00). Before the experiments the animals were acclimated to these conditions for a period of at least one week. The animals were fed standard rodent chow and water ad libitum.

[0046] Surgical drugs and reagents were purchased from Bachem, (California, USA). Human recombinant soluble TNF-R1 molecule used in this study has a sequence corresponding to segment 20-180 of human TNF-R1 (Niphar et al., 1990) and was prepared in CHO cells and provided by Interpharm Laboratories Ltd. (Israel) under the name of r-hTNF-1.

[0047] Material. General cell culture material was purchased from Gibco BRL Life Technologies (Paisley, UK). Estradiol-17 $\beta$  RM 10 was purchased from DPC (Los Angeles, CA, USA). Inokatrim was purchased from Vitrec (Carre, FR). <sup>51</sup>Cr-medium chromate was purchased from NEN Dupont (Boston, MA, USA). Rompus™ was purchased from Bayer (Leverkusen, DE). SRIK sutura 7.0 was purchased from Ethicon (Somerville, NJ).

[0048] Experimental model of endometriosis in the rat. To explore the effects of the recombinant soluble TNF-R1 in endometriosis, as previously described experimental model (Jones, 1987) was used with minor modifications. Under Inokatrim/Rompus™ anaesthesia, an autograft fragment of endometrial tissue (1 cm in length) was resected from the right uterine horn and placed in PBS at 37°C. The uterine segment was opened by a longitudinal incision, and a 5x5 mm section was transplanted, without removing the myometrium, onto the inner surface of the abdominal wall using non-absorbable suture, 4/0 at four corners.

[0049] Exploration of the size effects in the experimental model of endometriosis. Experimental endometriosis was surgically induced in engrafted rats as reported above. In addition, another group of rats similarly had a fragment of one uterine horn removed but a 5x5-mm square of fat (surrounding the uterine horn) was transplanted (sham-operated group). One further group of rats, not undergoing any surgical procedure, was kept as a normal control group. Three weeks after the induction of endometriosis, the animals underwent a second laparotomy (pre-treatment laparotomy) to evaluate the size and viability of the engrafted tissue. The uterine segment (approximately 1 cm in width) was measured using a calliper and weighed. Tissue samples showing viable engraftments were assigned to the designated treatment groups as reported in Table I, so that at the end of the experiment, six animals/group for the first sacrifice time-point and five animals/group for the second sacrifice time-point were obtained. The treatments were started after a 1-week recovery period. The control groups received saline alone; another group received three subcutaneous injections of 2 mg/kg Anti<sub>2</sub> every 3 days with a regimen previously shown to suppress ovarian and hypothalamic activity (Shupe et al., 1990). A further received 10 mg/kg recombinant soluble TNF-R1, divided into two daily doses over a 1-week period.

Table I

Endometrium Transplantation	Treatment	Days of treatment(1)	Days of Sacrifice(1)
No	Saline	28 through 34	36, 43
Sham-operated	Saline	28 through 34	36, 43
Yes	Saline	28 through 34	36, 43
Yes	Anti <sub>2</sub> (2 mg/kg)	25, 31, 34,	38, 43
Yes	Recombinant soluble TNF-R1 (10 mg/kg in two daily doses)	28 through 34	36, 43

(1) Post the day of surgical engraftment (day 13).

At the designated sacrifice time-points (2 and 9 days after the last treatment, i.e. 36 and 43 days after surgical engraftment), the animals were anaesthetized; blood samples were collected from the abdominal aorta, sera were separated and stored at -20°C until analysis for estradiol-17 $\beta$  level determination. Spleens were excised for measurement of the Natural Killer (NK) activity. The surface area of endometriosis-tissue foci was measured at each sacrifice time-point, in

order to normalize data, the percent variation versus the pre-treatment laparotomy value was calculated by the formula:

$$\frac{(X_0 - X)}{X_0} \times 100$$

where  $X_0$  is the size at time of pre-treatment laparotomy and  $X$  is the size at the time of sacrifice. The mean value of percent variation in each group was then computed.

[0050] NK activity determination. Murine lymphocytes in culture were harvested during the exponential growth phase and labelled with chromium (RPMI 1640 containing phosphate-buffered medium and 10% heat-inactivated fetal calf serum). The cell culture was incubated with 100  $\mu$ Ci of <sup>51</sup>Cr-epidium chromate at 37 °C, 5% CO<sub>2</sub> for 2 hours. Cells were then washed 3 times with 10 ml of assay medium, resuspended at the desired concentration (2 x 10<sup>6</sup>/ml) and serial dilutions were carried out in the assay medium in triplicate wells of a U-bottom 96-well plate prior to the addition of <sup>51</sup>Cr-labelled target cells. <sup>51</sup>Cr-labelled target cells (5 x 10<sup>3</sup>) were added to each well of the assay plate and three effector-to-target ratios (200:1, 100:1 and 50:1) were assayed for each sample. The plate containing the effector-to-target cell mixture was centrifuged at 200 x g for 4 min, and then incubated at 37 °C, 5% CO<sub>2</sub> for 4 hours. After an additional centrifugation of the plate at 200 x g for 4 min, 20  $\mu$ l of the supernatant from each well was transferred to a glass fiber filter and the associated radioactivity was evaluated by a  $\beta$ -counter.

The percentage of lysis was calculated as follows:

$$\frac{\text{cpm}_{\text{sample}} - \text{cpm}_{\text{control}}}{\text{cpm}_{\text{sample}} + \text{cpm}_{\text{control}}} \times 100$$

where:

cpm<sub>sample</sub> = mean <sup>51</sup>Cr release in the presence of effector cells

cpm<sub>control</sub> = mean <sup>51</sup>Cr release of target cells in the presence of culture medium

cpm<sub>base</sub> = mean <sup>51</sup>Cr release of target cells in the presence of 1% Triton-X100.

[0051] Estradiol-17 $\beta$  determination. Serum estradiol-17 $\beta$  concentrations were determined using a commercially available kit to quantify estradiol in serum with an extraction step (DPC, Los Angeles, CA, USA). Briefly, <sup>125</sup>I-labelled estradiol competes with estradiol in the serum sample for antibody sites. After incubation, separation of bound from free estradiol was achieved by decanting. The tube was then counted in a gamma counter (ICG-Pharmacia Wallac), the counts being inversely related to the amount of estradiol present in the serum sample. The quantity of estradiol in the samples was determined by comparing the counts to a calibration curve. The antisera is highly specific for estradiol, with a relatively low cross-reactivity to other naturally occurring steroids. Samples from the same experimental session were analyzed in a single immunoassay.

[0052] Statistical analysis. Statistical significance of the differences observed among the treatment groups was evaluated using the ANOVA present in the Statgraphics Plus® software (Version 1.4). The Tukey's multiple range test ( $P < 0.05$ ) was performed.

#### Results

##### Exploration of Recombinant Soluble TNF-R1 effects in experimental endometriosis.

[0053] Successful growth and development of surgically transplanted endometrial tissue in the rat has offered a research model that has been used to study some of the aspects of endometriosis that cannot be adequately investigated in humans (Dudley et al., 1992). Previous studies in rat experimental endometriosis indicate that Anti<sub>2</sub> works properly as a positive control (Shupe et al., 1990). In the present example the effect of Anti<sub>2</sub> was compared, in term of dimension of engraftment size before and after treatment, with the ones obtained using recombinant soluble TNF-R1, as summarized in Table II.

Table 8

Treatment	Dose (mg/kg)	Observation Time After Last Treatment (day)	Mean±SEM Pre-treatment values (cm <sup>2</sup> )	Mean±SEM Post-treatment values (cm <sup>2</sup> )
Saline	-	2	1.45±0.41	1.18±0.15
Soluble TNF-R1	10	2	1.43±0.38	0.77±0.09
Anti-αIE	2	2	1.43±0.34	0.08±0.02
Saline	-	9	1.28±0.24	0.97±0.11
Soluble TNF-R1	10	9	1.42±0.38	0.54±0.19
Anti-αIE	2	9	1.41±0.32	0.19±0.09

[0053] The results are expressed in Figure 1 as the mean percentage inhibition of grafted endometrium fragments (calculated as described above).

[0054] Anti-IE was effective in reducing the size of the endometriotic-like foci (Fig. 1), inducing an almost complete (94 % and 88 % compared to the original dimension, respectively) and statistically significant ( $p<0.05$ , ANOVA and Tukey's test) remission at both observation time-points after discontinuation of treatment. The 1-week treatment with human recombinant soluble TNF-R1 (10 mg/kg, two daily doses) resulted in a significant size reductions (33 % and 64 % compared to the original dimension) of the endometriotic-like foci at both observation time-points but statistically significant ( $p<0.05$ , ANOVA and Tukey's test) only at day 9. Engraftments were not observed in the sham-operated animals at any time.

#### NK cell activities evaluation

[0055] NK cell activity was evaluated by *in vitro* tests with spleen cells against YAC cells did not show any difference among groups (Fig. 2), similarly to what has been observed in humans, where no difference in endometrial cytotoxicity and NK cell activity was found in animals with and without endometriosis (DHooghe et al., 1995). This finding is in contrast with human data where depressed NK activity in patients with endometriosis has been reported with a significant correlation between reduced peritoneal NK activity and severity of endometriosis (Oosterlynck et al., 1992).

#### Breast estradiol-17 $\beta$ evaluation

[0056] The serum estradiol-17 $\beta$  concentrations were measured by radioimmunoassay at both observation time-points. A significant difference was observed in the Anti-IE-treated group as compared to the untreated controls at the second observation time-point. No statistical significant differences were observed for recombinant soluble TNF-R1 when compared to controls (Fig. 3;  $p>0.05$ , ANOVA and Tukey's test).

#### Conclusions

[0057] In the rat experimental model of endometriosis, administration of a TNF antagonist, the soluble form of TNF-R1, provides, for the first time, a clear evidence of the potential effectiveness of cytokine-based, non hormone-related treatment of this pathological condition. Thus, TNF antagonists represent an alternative to the existing medical treatments in terms of reduced side effects. These results assess the use of TNF antagonists in the treatment of endometriosis-related infertility.

[0058] Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the inventions described herein. These and all other equivalents are intended to be encompassed by the following claims.

#### Reference List

- [0059] Arici A., Tazkia, S.I., Atzar, E., Kliman, H.J., and Oliva, D.L., 1996a, Mol.Hum.Reprod. 2, 40-45.
- [0060] Arici A., Oral, E., Bubamaz, O., Dulbe, A., Oliva, D.L., and Jones, E.E., 1996b, Fertil.Steril. 65, 603-607.
- [0061] Arici A., Oral, E., Atzar, E., Tazkia, S.I., and Oliva, D.L., 1997, Fertil.Steril. 67, 1065-1072.

composition for the treatment and/or prevention of endometriosis, wherein said TNF antagonist is a sequestering antagonist or a signaling antagonist.

2. Use of a TNF antagonist together with a pharmaceutically acceptable carrier in the preparation of a pharmaceutical composition to improve the implantation and fertility rate by reducing endometriotic lesions, wherein said TNF antagonist is a sequestering antagonist or a signaling antagonist.
3. The use of Claims 1 or 2 wherein the sequestering antagonist is a receptor molecule, derivative or a fragment thereof which binds to TNF.
4. The use of Claim 3 wherein the receptor molecule is selected from the group consisting of: TNF-R1 and TNF-R2.
5. The use of Claim 3 wherein the receptor molecule is the extracellular domain of TNF-R1.
6. The use of Claim 3 wherein the receptor molecule is human soluble recombinant TNF-R1.
7. The use of Claim 3 wherein the receptor molecule is a TNF receptor multimeric molecule or a functional portion thereof.
8. The use of Claim 7 wherein the TNF receptor multimeric molecule comprises all or a functional portion of two or more extracellular domains of TNF receptors linked via one or more polypeptide linkers.
9. The use of Claim 3 wherein the receptor molecule is an immunoreceptor fusion molecule or a functional portion thereof.
10. The use of Claim 9 wherein the immunoreceptor fusion molecule comprises all or a functional portion of TNF receptor and an immunoglobulin chain.
11. The use of Claims 1 or 2 wherein the sequestering antagonist is an anti-TNF antibody or a fragment thereof.
12. The use of Claim 11 wherein the monoclonal antibody is selected from the group consisting of: a chimeric monoclonal antibody, a humanized monoclonal antibody or fragment thereof.

#### Patentsprüche

1. Verwendung eines TNF-Antagonisten zusammen mit einem pharmazeutisch annehmbaren Träger in der Herstellung einer pharmazeutischen Zusammensetzung für die Behandlung und/oder Verhütung von Endometriose, wobei dieser TNF-Antagonist ein sequestrierender Antagonist oder ein signalisierender Antagonist ist.
2. Verwendung eines TNF-Antagonisten zusammen mit einem pharmazeutisch annehmbaren Träger in der Herstellung einer pharmazeutischen Zusammensetzung, um die Implantations- und Fruchtbarkeitsrate durch Verringerung von endometriotischen Läsionen zu verbessern, wobei dieser TNF-Antagonist ein sequestrierender Antagonist oder ein signalisierender Antagonist ist.
3. Verwendung des Anspruchs 1 oder 2, wobei der sequestrierende Antagonist ein Rezeptormolekül, dessen Abkömmling oder Fragment ist, das an TNF bindet.
4. Verwendung des Anspruchs 3, wobei das Rezeptormolekül aus der Gruppe ausgewählt ist, die aus TNF-R1 und TNF-R2 besteht.
5. Verwendung des Anspruchs 3, wobei das Rezeptormolekül der extrazelluläre Bereich von TNF-R1 ist.
6. Verwendung des Anspruchs 3, wobei das Rezeptormolekül körpereigener rekombinanter TNF-R1 ist.
7. Verwendung des Anspruchs 3, wobei das Rezeptormolekül ein multimerses TNF-Rezeptormolekül oder ein funktioneller Abschnitt davon ist.

8. Verwendung des Anspruchs 7, wobei das multimerses TNF-Rezeptormolekül die Gesamtheit oder einen funktionellen Abschnitt von zwei oder mehr extrazellulären Bereichen von TNF-Rezeptoren umfasst, die über ein oder mehrere Polypeptid-Linker verbunden sind.

9. Verwendung des Anspruchs 3, wobei das Rezeptormolekül ein Immunrezeptor-Fusionemolekül oder ein funktioneller Abschnitt davon ist.

10. Verwendung des Anspruchs 8, wobei das Immunrezeptor-Fusionemolekül die Gesamtheit oder einen funktionellen Abschnitt des TNF-Rezeptors und eine Immunglobulinkette umfasst.

11. Verwendung der Ansprüche 1 oder 2, wobei der sequestrierende Antagonist ein Anti-TNF-Antikörper oder ein Fragment davon ist.

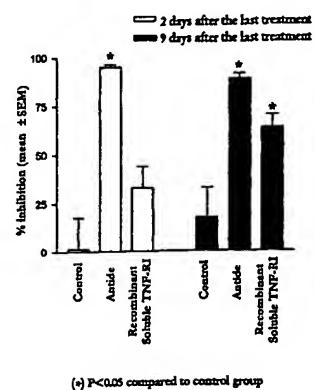
12. Verwendung des Anspruchs 11, wobei der monoklonale Antikörper aus der Gruppe ausgewählt ist, die aus einem monoklonalen chimaeren Antikörper, einem monoklonalen humanisierten Antikörper oder einem Fragment davon besteht.

#### Revendications

1. Utilisation d'un antagoniste de TNF avec un support pharmaceutiquement acceptable dans la préparation d'une composition pharmaceutique pour la traitement et/ou la prévention de l'endométriose, dans laquelle ledit antagoniste TNF est un antagoniste par séquestration ou un antagoniste par signalisation.
2. Utilisation d'un antagoniste de TNF avec un support pharmaceutiquement acceptable dans la préparation d'une composition pharmaceutique pour améliorer le taux d'implantation et de fertilité en réduisant les lésions endométriotiques, dans laquelle ledit antagoniste TNF est un antagoniste par séquestration ou un antagoniste par signalisation.
3. Utilisation selon les revendications 1 ou 2 dans laquelle l'antagoniste par séquestration est une molécule/réceptrice, un dérivé ou un fragment de celle-ci qui se lie au TNF.
4. Utilisation selon les revendications 3 dans laquelle la molécule/réceptrice est choisie parmi le groupe constitué de : récepteur TNF-R1 et récepteur TNF-R2.
5. Utilisation selon les revendications 3 dans laquelle la molécule/réceptrice est le domaine extracellulaire du récepteur TNF-R1.
6. Utilisation selon la revendication 3 dans laquelle la molécule/réceptrice est un récepteur TNF-R1 recombinant soluble humain.
7. Utilisation selon la revendication 3 dans laquelle la molécule/réceptrice est une molécule d'un récepteur TNF multimerique ou une partie fonctionnelle de celle-ci.
8. Utilisation selon la revendication 7 dans laquelle la molécule d'un récepteur TNF multimerique comprend tout ou une partie fonctionnelle de deux ou plusieurs domaines extracellulaires des récepteurs TNF liés via un ou plusieurs liens polypeptidiques.
9. Utilisation selon la revendication 3 dans laquelle la molécule/réceptrice est une molécule de fusion immunoréceptrice ou une partie fonctionnelle de celle-ci.
10. Utilisation selon la revendication 3 dans laquelle la molécule de fusion immunoréceptrice comprend tout ou une partie fonctionnelle du récepteur TNF et une chaîne d'immunoglobulines.
11. Utilisation selon la revendication 1 ou la revendication 2 dans laquelle l'antagoniste par séquestration est un anticorps anti-TNF ou un fragment de celui-ci.
12. Utilisation selon la revendication 11 dans laquelle l'anticorps monoclonal est sélectionné parmi le groupe constitué

de : un anticorps monoclonal chimérique, un anticorps monoclonal humainisé ou un fragment de cellu-cl.

Figure 1



(\*) P<0.05 compared to control group

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Figure 2

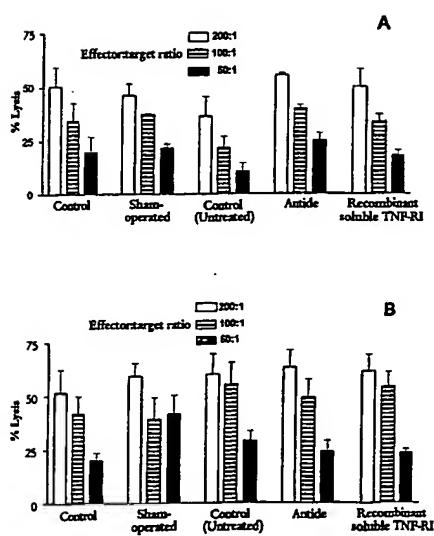
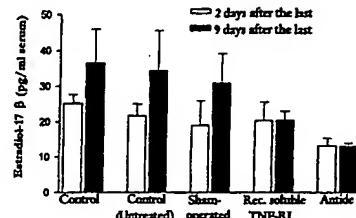


Figure 3



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